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VDAC1 serves as a mitochondrial binding site for hexokinase in oxidative muscles

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Abstract

Voltage-dependent anion channels (VDACs), also known as mitochondrial porins, are the main pathway for metabolites across the mitochondrial outer membrane and may serve as binding sites for kinases, including hexokinase. We determined that mitochondria-bound hexokinase activity is significantly reduced in oxidative muscles (heart and soleus) in *vdac1*$^{-/-}$ mice. The activity data were supported by western blot analysis using HK2 specific antibody. To gain more insight into the physiologic mean of the results with the activity data, VDAC deficient mice were subjected to glucose tolerance testing and exercise-induced stress, each of which involves tissue glucose uptake via different mechanisms. *vdac1*$^{-/-}$ mice exhibit impaired glucose tolerance whereas *vdac3*$^{-/-}$ mice have normal glucose tolerance and exercise capacity. Mice lacking both VDAC1 and VDAC3 (*vdac1*$^{-/-}$/*vdac3*$^{-/-}$) have reduced exercise capacity together with impaired glucose tolerance. Therefore, we demonstrated a link between VDAC1 mediated mitochondria-bound hexokinase activity and the capacity for glucose clearance.

Keywords: Mitochondrial outer membrane; Muscle; Porin; Hexokinase; Glucose clearance; Exercise capacity

1. Introduction

Two groups of kinases are found in the mitochondria: energy consuming kinases at the outer surface (e.g., acyl-CoA-synthetase, hexokinase and glycerol kinase) and energy transmitting kinases in the intermembrane space (e.g., adenylate kinase, creatine kinase and nucleoside diphosphate kinase). By converting glucose to glucose 6-phosphate, hexokinase (HK) helps maintain the glucose concentration gradient that results in the movement of glucose into cells through the facilitative glucose transporters. In skeletal muscle and heart, HK2 is the major hexokinase isoform. The enzyme is either located in the cytosol or associated with the mitochondria, a property termed "ambiquitous" [1,2]. Skeletal muscle is a primary site for both insulin- and exercise-stimulated glucose disposals. In rodent and human skeletal muscle, HK2 is subject to regulation since insulin and muscle contraction increases the expression of HK2 but not HK1 [3–5]. During resting conditions, the transport step exerts the most control in regulating muscle glucose uptake, as GLUT1 or GLUT4 overexpression augments basal muscle glucose uptake [6–11]. Hyperinsulinemia and exercise shift the control of muscle glucose uptake so that glucose phosphorylation is a more important determinant of the rate of this process. Indeed, transgenic mice overexpressing HK2 required 25% more glucose to maintain euglycemia during hyperinsulinemic experiments. When exercise-induced glucose uptake was assessed, muscle 2-deoxy-[^{3}H] glucose accumulation was significantly greater [12]. Recently, it has been reported that in mice heterozygous for a HK2 null allele, exercise-stimulated glucose uptake in oxidative muscles is impaired [13]. This impairment is critically dependent on metabolic activity and associated glucose flux. Whereas muscle glucose uptake has been separated into three sequential steps: delivery of glucose from the blood to the muscle, transport across the sarcolemma and irreversible phosphorylation to glucose-6-phosphate; the possibility that mitochondria-bound hexokinase activity could constitute an additional control step has not been definitely addressed.

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Voltage-dependent anion channels (VDACs) are pore-forming proteins found in the mitochondrial outer membrane (MOM) of all eukaryotes [14]. In mammals, three VDAC isoforms have been characterized: VDAC1, VDAC2 and VDAC3 [15–20]. We have previously reported that all three VDAC isoforms are expressed in wild type muscles. It has previously been demonstrated that the pore protein and the hexokinase binding protein from the MOM of rat liver mitochondria are identical [21,22] and that, *in vitro*, hexokinase and VDAC form complexes [23]. It is believed that, *in vivo*, kinase:VDAC interaction facilitates the access of kinases to ATP and overcomes the restriction that the MOM exerts on the permeability for the metabolites [24–26].

Mice lacking the expression of VDAC1 (*vdac1*$^{-/-}$) or VDAC3 (*vdac3*$^{-/-}$) have been generated by gene targeting in embryonic stem cells [27–29]. Double mutant mice (*vdac1*$^{-/-}$/*vdac3*$^{-/-}$) have been obtained by intercrossing *vdac1*$^{+/-}$ and *vdac3*$^{+/-}$ mice. Although *vdac1*$^{-/-}$ and double mutant mice exhibit partial lethality *in utero*, the live born mice are viable and do not exhibit any cardiovascular structural abnormalities. However, *vdac1*$^{-/-}$ and *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice are growth retarded. We expected that the absence of VDAC will alter the amount of mitochondria-bound hexokinase, leading to altered substrate utilization. We therefore measured total and mitochondria-bound hexokinase activities. Two approaches were used to indirectly examine substrate utilization in VDAC deficient mice: we challenged VDAC deficient mice by glucose tolerance testing and by using an open circuit indirect calorimetry treadmill system.

2. Experimental procedures

2.1. Animals

All experiments using animals were conducted using protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Male and female *vdac1*$^{-/-}$, *vdac3*$^{-/-}$ and *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice were used in the present study. Mice were CD1 (*vdac1*$^{-/-}$ mice), C57BL6 (*vdac3*$^{-/-}$ mice) or C57BL6/129SvEv (*vadc1*$^{-/-}$ mice in the growth experiment and *vac1*$^{-/-}$/*vdac3*$^{-/-}$ mice) strains. Six months old male and female wild type littermates served as age-matched controls. Genotyping of the mice was performed via PCR analysis [27–29]. Isolation of mouse tail DNA was performed with the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions.

2.2. Hexokinase activity

Fresh heart and soleus were homogenized (50 mg/ml) using an Ultra-Turax Polytron in 5 mM HEPES buffer (pH 8.0) containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1% Triton X-100. Homogenates were allowed to sit on ice for 1 h then centrifuged at 2,500 rpm, 5 min. Total hexokinase activity was measured on the supernatant in the presence of 20 mM HEPES (pH 8.0), 20 mM glucose, 0.5 mM DTT, 5 mM $MgCl_2$, 1.5 mM ATP, 0.5 mM NADP and 5 U glucose 6-phosphate dehydrogenase. A coupled enzyme system was used to follow the rate of NADPH formation at 340 nm, 25 °C [30]. To measure the mitochondria-bound hexokinase activity, the same assay was performed on permeabilized muscle fibers, as previously described [31]. The sarcolemmal membrane was permeabilized as previously described for mice [27,32]. Fiber bundles from the endocardium and soleus were placed in a cooled solution S (pH 7.1) containing 1.9 mM CaK_2EGTA, 8.1 mM K_2EGTA, 9.44 mM $MgCl_2$, 3 mM KH_2PO_4, 2.5 mM Na_2ATP, 15 mM phosphocreatine, 0.5 mM DTT, 53 mM potassium methanesulfonate, 20 mM imidazole, 20 mM taurine and incubated in the presence of 50 μg/ml saponin with mild agitation for 30 min, 4 °C. The bundles were then washed twice in solution S without PCr and Na_2ATP for 10 min. The skinned fibers were then weighed and homogenized as described above. The hexokinase activity determined on the skinned fibers homogenates represents the mitochondria-bound hexokinase activity [31].

2.3. Western blotting

Five to ten μg of the sample homogenates from the hexokinase assay were separated using a 12% ready-gel (Bio-Rad) and transferred to polyvinylidene difluoride membrane (Bio-Rad) using a Bio-Rad Trans-Blot system. A prestained, broad range standard was used as a molecular weight marker (161–0372, Bio-Rad). The membranes were blocked in 5% milk/1× Tris Borate SDS (TBS), 0.05% Tween and incubated with an affinity column purified rabbit polyclonal antibody raised against the rat HK2 (kind gift from Dr. John E. Wilson) [33] for 2 h at room temperature. A rabbit polyclonal antibody for actin was used as a loading control (A2066, Sigma). The dilution was 1:2,000 for both HK2 and actin. A mouse anti-rabbit IgG HRP conjugate (Sigma) was used at a final dilution of 1:10,000. The membranes were developed using an ECL plus Western Blotting Detection System (RPN 2132, Amersham Biosciences) and developed using Blue X-ray Film BX (F-BX810, Phenix Research Products). Protein was determined with the BCA reagent (#23225, Pierce) with BSA as standard.

2.4. Glucose tolerance testing

Mice from control and VDAC deficient groups were fasted for 16 h. D-Glucose (1.2 mg/g weight) was injected into the peritoneum of conscious mice. Blood was obtained from the tail at 0, 30, 60, 90 and 120 min after injection and glucose concentration was determined with an Elite glucose meter (Glucometer Elite, Bayer).

2.5. Incremental treadmill exercise

The treadmill apparatus (Columbus Instruments, Columbus, Ohio, USA) employs a moving belt encased in a Plexiglas enclosure, assuring that the mouse remains on the belt and allowing for body mass-corrected oxygen consumption and carbon dioxide production measurements. The apparatus is equipped with an electrified grid at the rear of the belt to provide motivation. We used a protocol similar to the one used by Graham et al. [34], with some minor changes. After an initial ~20 min baseline, the mice were subjected to a ~20 min exercise protocol, under constant supervision, during which the workload was increased every 90 s by increasing the belt speed and/or the belt incline. We started the exercise with 5 m/min at 0° incline and ended with 17 m/min at 25° incline (Fig. 5A). We defined fatigue when the animal sat on the electric grid for at least 10 s. At this point, we stopped the electric shock and allowed the animal to recover. We measured the percentage of completion of the protocol in controls and VDACs deficient mice. To acclimate to the system, each mouse was subjected to a 10 min run (5 m/min, 0° incline) the day before the actual experiments. Gas exchange was measured using a computer-controlled open-circuit indirect calorimeter (Oxymax, Columbus Instruments) with an air flow of 0.5 l/min at 22 °C. Gas samples were dried, measured and averaged at 90 s intervals using Oxymax software. Calibration of the O_2 and CO_2 gas analyzers was performed daily using a standard gas mixture (0.5% CO_2–20.5% O_2–balanced with N_2).

2.6. Plasma glucose and lactate determination

Blood (~ 200 μl) was collected from the mouse tail vein. Serum glucose and lactate were determined using a glucose/lactate analyzer (2300 STAT PLUS, YSI).

2.7. Statistical analysis

A Student *t*-test was used to analyze data. $P<0.05$ was set as level of significance.

ANOVA test was used to compare the body weight between knock-out and control groups. $P<0.05$ was set as level of significance.

3. Results

3.1. Mice

To determine whether the absence of VDAC affects growth, we followed the growth rate in control and VDAC deficient mice on mixed or inbred backgrounds fed ad libitum. The adult *vdac1*$^{-/-}$ mice exhibited mild growth retardation on mixed background that did not reach the level of significance. Growth retardation was more pronounced in *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice when compared to littermate controls ($P<0.05$) (Fig. 1A). There was no growth deficiency in *vdac3*$^{-/-}$ mice maintained on a C57BL6 background (Fig. 1B). *vdac2*$^{-/-}$ mice were not available for study.

3.2. Hexokinase activity

Due to the difference in background, we represented the activity in the different VDAC deficient group as a percentage of the corresponding wild type control. By measuring the hexokinase activity in heart and soleus from wild type and VDAC deficient mice, we observed a significant decrease in the mitochondria-bound hexokinase activity in *vdac1*$^{-/-}$ mice, whereas in *vdac3*$^{-/-}$ mice the activity is indistinguishable from wild type controls (Fig. 2). This decrease can be directly correlated with the absence of VDAC1, since the total hexokinase activity is not significantly different from the wild type.

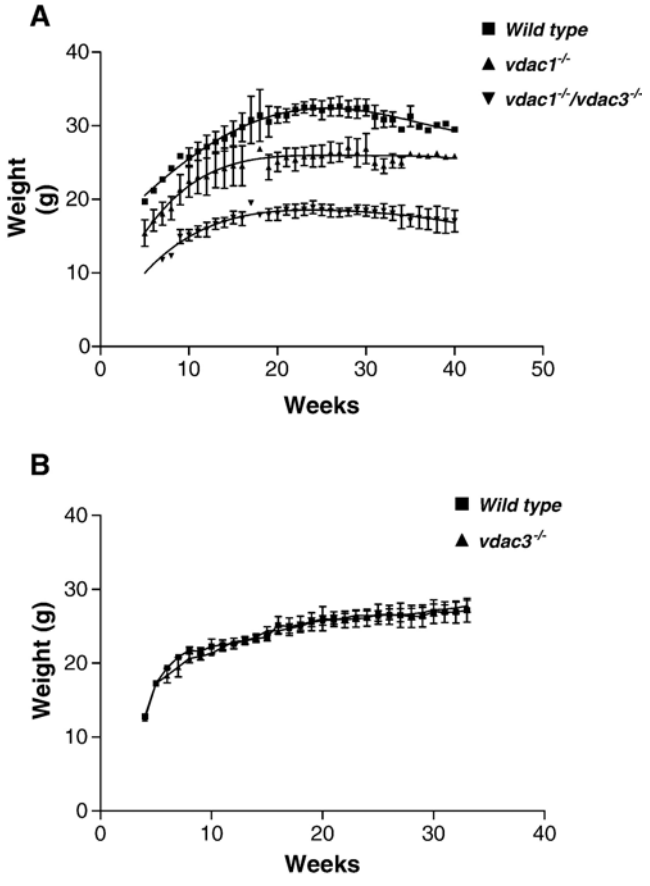

Fig. 1. Weight curves of wild type and different VDAC deficient mice. Mice, fed ad libitum, were weighed weekly for up to ~40 weeks. A) Male mice on C57/129SvEv background (wild type square $n=6$, *vdac1*$^{-/-}$ triangle $n=5$, *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ inversed triangle, $P<0.05$ vs. wild type, $n=3$). B) Male mice on C57BL6 inbred background (wild type square $n=3$, *vdac3*$^{-/-}$ triangle $n=4$).

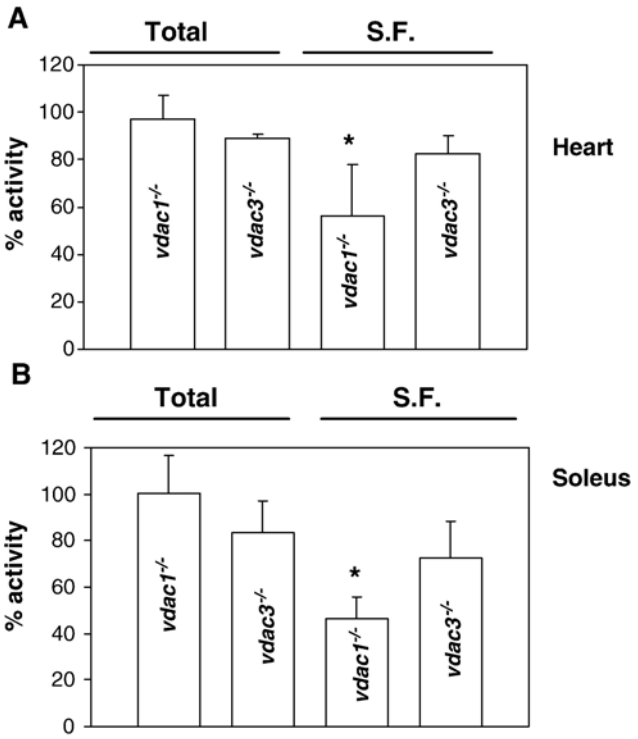

Fig. 2. Total and mitochondria-bound hexokinase activity from different muscle groups: (A) in heart, (B) in soleus. The activities in different VDAC deficient muscles are represented as percentage of their corresponding wild type activity. The different values represent the Mean±SD, $n=6$, *$P<0.05$ vs. wild type using a Student *t*-test. Total and S.F. represent sample homogenate from total and skinned fibers preparations.

3.3. Western blotting

To verify that the decrease in hexokinase activity correlates with a decrease in mitochondria-bound hexokinase protein, aliquots from wild type and VDAC deficient skinned fibers homogenates used in the hexokinase activity assay were analyzed by using a HK2 specific antibody. The enzymatic activity data correlated with the protein content in the skinned fibers prepared from *vdac1*$^{-/-}$ heart that shows a decrease in the amount of HK2 (Fig. 3A). Due to the size of the soleus muscle from *vdac1*$^{-/-}$ mice, we could not perform the western blot analysis on this muscle. HK2 amount in *vdac3*$^{-/-}$ muscles is indistinguishable from wild type muscles (Fig. 3B).

3.4. Glucose tolerance

While hexokinase binding to mitochondria has been reported repeatedly, the question of whether the hexokinase binding to

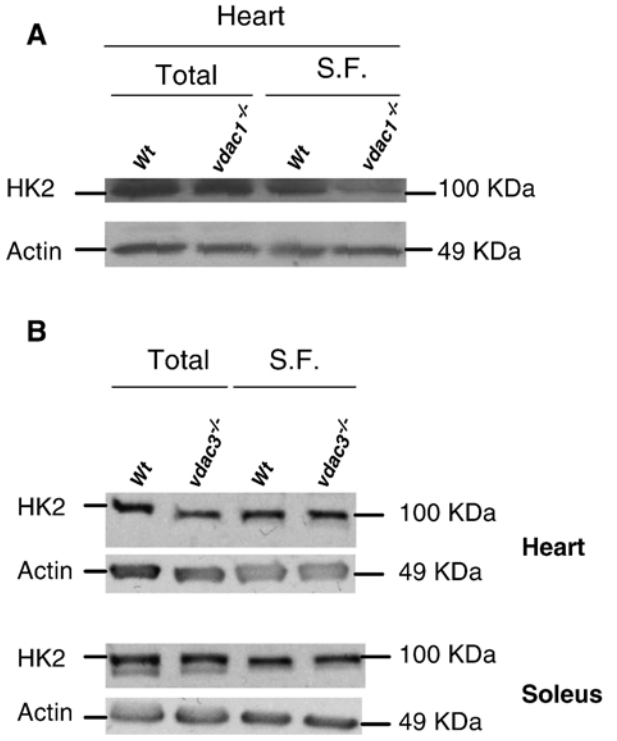

Fig. 3. Western blots corresponding to the total and mitochondria-bound hexokinase from wild type and different VDAC deficient muscles; (A) in *vdac1*$^{-/-}$ heart, (B) in *vdac3*$^{-/-}$ heart and soleus. The different blots have been probed with HK2 specific antibody and an anti-actin as loading control as described in Experimental procedures. Total and S.F. represent sample homogenate from total and skinned fibers preparations.

mitochondria could constitute an additional regulatory step for muscle glucose utilization in vivo has not previously been rigorously addressed. In mammals, skeletal muscle is a primary target for the stimulation of glucose transport by a variety of activators. These include insulin and stimuli that increase energy demand such as exercise. To determine whether VDAC deficient mice have an altered glucose clearance, we measured glucose tolerance of different VDAC deficient groups. While *vdac3*$^{-/-}$ mice exhibit a glucose tolerance curve indistinguishable from wild type controls, *vdac1*$^{-/-}$ mice have an impaired glucose tolerance (Fig. 4). *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice have also impaired glucose tolerance (data not shown).

3.5. Treadmill exercise

Treadmill exercise is typically used for inducing graded physiologic stress in evaluating the response of various organ systems [34,35]. While single mutant mice exhibited an exercise tolerance indistinguishable from control mice, double mutant mice showed a reduced capacity for exercise (Fig. 5B). The contribution of anaerobic metabolism to overall energy production during exercise can be estimated by calculating the respiratory exchange ratio (ratio of VCO_2 to VO_2 or RER). Under increasing workloads none of the groups tested reached a RER value higher than 1, therefore none of these groups reached their maximum aerobic capacity within the context of the present study. However, the *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ group had a significantly lower RER at the end of the exercise period (Fig. 6A), due to a relatively higher rate of O_2 consumption (data not shown). This result is consistent with a relatively higher contribution of lipids to overall metabolism in the double mutant mice [36].

Lactate levels were not significantly different between the control and the double mutant mice, both at rest (4.09±0.36 mM in wild type vs. 4.52±0.32 mM in *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice) and after the exercise (13.3±0.82 mM in wild type vs. 12.0±1.09 mM in *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice). However, although the serum glucose level at rest was indistinguishable between the control and the double mutant mice (9.89±0.45 mM in the control vs. 8.94±0.91 mM in the double mutant mice), after exercise the serum glucose concentration was significantly higher in the double mutant mice (12.0±0.77 mM in *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice versus 8.94±0.91 mM in control, $P<0.05$) (Fig. 6B). Taken together, these results might suggest that *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice utilize less glucose when exercised.

4. Discussion

Since it has long been held that a VDAC is the mitochondrial receptor for hexokinase, we speculated that the absence of VDAC would alter mitochondria-bound hexokinase. If mitochondria-bound hexokinase activity plays a regulatory role in

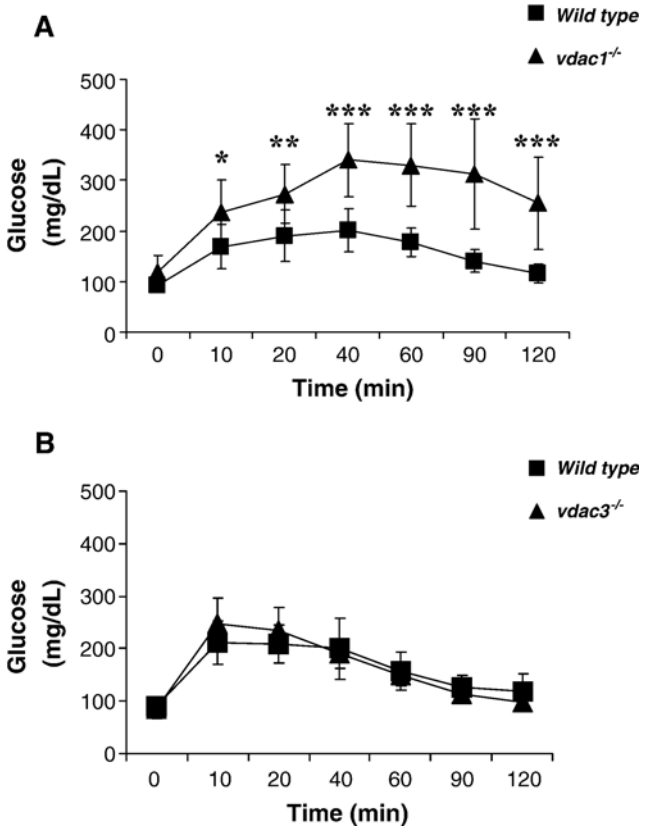

Fig. 4. Glucose tolerance curve in wild type (square) and different VDAC deficient (triangle) groups. (A) Wild type vs. *vdac1*$^{-/-}$ mice ($n=8$ for each groups). (B) Wild type vs. *vdac3*$^{-/-}$ mice ($n=8$ for each group). The different time point values represent the Mean±SD, *$P<0.05$ vs. wild type, **$P<0.01$ vs. wild type, ***$P<0.001$ vs. wild type.

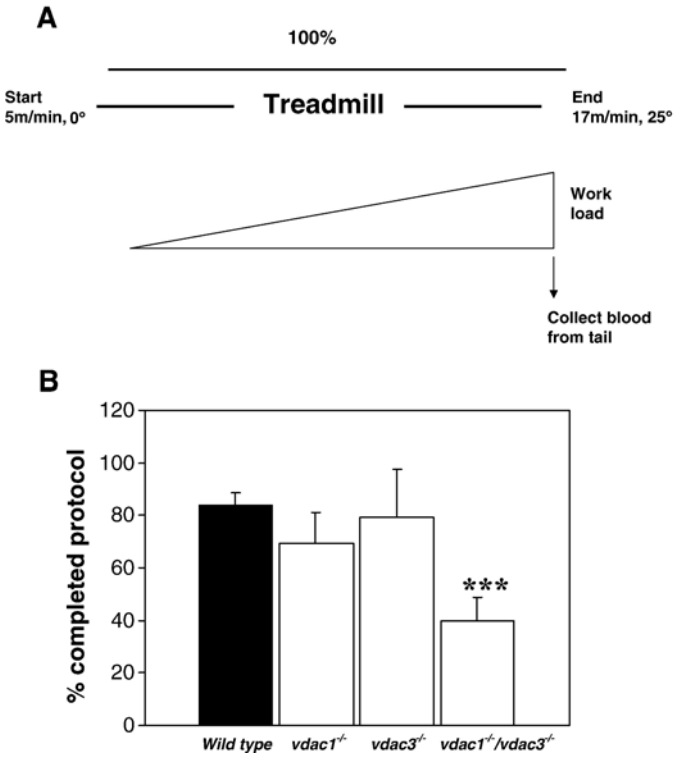

Fig. 5. Exercise testing in wild type and different VDAC deficient mice. (A) Scheme of the treadmill test. (B) Exercise tolerance of wild type and different VDAC deficient mice. Mice were exercised as described in Experimental procedures until exhaustion, and then the percentage of completion of the test was determined. Black bar=wild type group ($n=12$); White bar=different VDAC deficient groups ($n\geq 6$). The different graphs represent Mean±SEM, ***$P<0.001$ vs. wild type using a Student t-test.

glucose uptake, we also hypothesized that the absence of VDAC will have an impact on glucose utilization under conditions of high glucose flux, e.g., glucose tolerance test and exercise. By using a skinned muscle fiber preparation, Parra et al. [31] established an assay for direct measurement of mitochondria-bound hexokinase activity in skeletal muscle. This method is more accurate to determine mitochondria-bound hexokinase because in the process of isolating mitochondria hexokinase may be lost during the different steps of isolation. The authors demonstrated that work induces mitochondrial binding and enhanced catalytic activity of hexokinase in fast-twitch muscles of the rat. By adapting the method to mouse muscle we have demonstrated that there is a decrease in the activity and the amount of mitochondria-bound hexokinase in VDAC1 deficient oxidative muscles.

It is well established that both insulin and exercise rapidly elevate glucose uptake into muscle, in part by signaling the translocation of glucose transporters from intracellular stores to the plasma membrane [37–42]. However, while insulin-stimulated GLUT4 translocation is mediated via the phosphatidylinositol 3-kinase (PI3-K) pathway, exercise-induced glucose uptake depends upon the AMP kinase signaling pathway to mobilize a distinct intracellular pool of GLUT4 [43,44]. By using insulin tolerance testing, we found similar insulin sensitivity in wild type and VDAC1 deficient mice (data not shown). More recently, studies done on conscious mouse have demonstrated that both insulin and exercise-stimulated muscle glucose uptake by GLUT4 are dependent on glucose phosphorylation capacity [45,46]. It has been also reported that in muscle composed of type II fibers (glycolytic), glucose delivery and glucose transport both serve to limit the rate of insulin-stimulated muscle glucose uptake. In contrast, in soleus muscle (composed of type I fibers), both glucose delivery and phosphorylation are determinants of the rate of glucose uptake [47]. Although glucose uptake has not been measured in the present study, our data with the glucose tolerance test and mitochondria-bound hexokinase activity in soleus do support these findings. Due to the difficulty obtaining glycolytic muscle from our mouse model, we restricted the study to the skeletal muscle soleus.

While skeletal muscle, liver and fat are the primary target tissues for glucose utilization post-prandially, a regulatory mechanism controlled principally by insulin, skeletal muscle is the principal site for glucose utilization during exercise, where glucose is metabolized by anaerobic glycolysis and aerobic oxidation to generate ATP. The data presented here do not address whether liver or adipose tissues play a significant role in the impaired glucose tolerance in this mouse model, and therefore further studies are needed to address this question. Indeed, using a proteomic approach to understand the functional

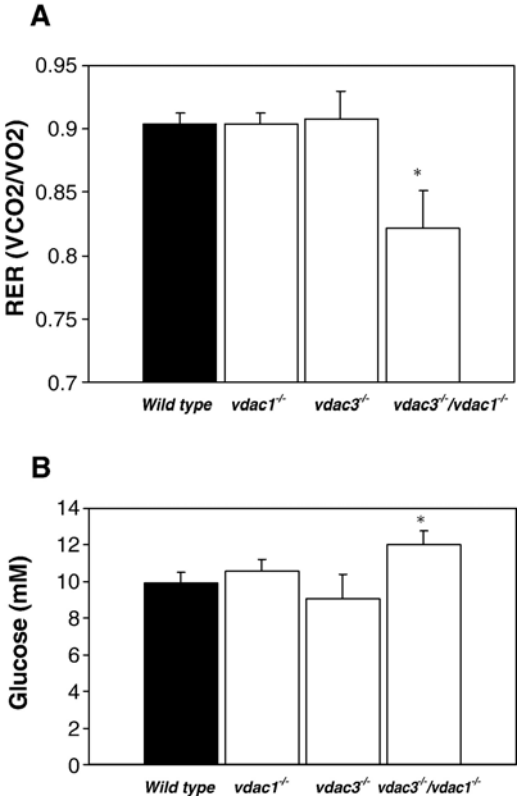

Fig. 6. The respiratory exchange ratio (RER) and serum glucose were significantly decreased and increased respectively at the end of the exercise in *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice. (A) RER of wild type (black bar) and different VDAC deficient mice (white bar) at the end of the exercise. (B) Serum glucose at the end of the exercise in wild type (black bar) and different VDAC deficient mice (white bar). The values represent the Mean±SEM, *$P<0.05$ vs. wild type using a Student t-test, $n\geq 5$.

role of the pro-apoptotic BH3-only molecule BAD and the consequences of loss of BAD, Daniel et al. [48] identified a multiprotein complex associated with mitochondria from mouse liver. This complex contained, amongst other proteins, glucokinase (HK4), the majority of which resides in the cytosol. They further demonstrated that BAD deficient mice exhibit glucose intolerance due to reduced HK4 mitochondrial association. They proposed that the complex regulates hepatic glucose uptake via directing a small fraction of HK4 to mitochondria for direct access to OXPHOS generated ATP. Whether the phenotype observed in *vdac1*$^{-/-}$ mice is in part related to reduced HK4 binding is currently under study. We also speculate that a similar defect may be observed in pancreatic beta cells.

The in vivo experiments in genetically manipulated mice are a useful method for studying organ-specific manipulations to selected proteins in the context of the whole animal. In mice, as in humans, the RER value can be used to determine substrate utilization [49]. An increase of the RER above 1.0 (anaerobic threshold) represents the point at which CO_2 production exceeds O_2 consumption and anaerobic metabolism begins to play a predominant role in energy production due to an imbalance in O_2 demand and O_2 delivery. Within the context of the present study, neither the control nor the mutant mice reached the anaerobic threshold. However, the double mutant mice reached fatigue well before the wild type mice and the RER at the end of exercise was significantly lower in the double mutant mice in comparison to the control mice. These data suggest that in the double mutant mice there is a larger contribution of lipid to overall energy production, and the exercise test uncovers a defect in the metabolic pathway of glucose utilization as supported by a higher serum glucose concentration at the end of the exercise. Due to the rarity of live borne double mutant mice, the hexokinase activity in the double mutant sample was represented by the mean of two different points. Nevertheless, this activity is lower than the lowest value observed for the *vdac1*$^{-/-}$ samples (data not shown). *vdac1*$^{-/-}$/*vdac3*$^{-/-}$ mice develop both impaired glucose and exercise tolerance. The fact that total hexokinase activity was decreased in the double mutant mice (data not shown) make it difficult to correlate the phenotype observed with altered mitochondria-bound hexokinase activity. However, the data demonstrate a link between the capacity of glucose phosphorylation and the stimulated state of glucose utilization. In a recent paper, Fueger et al. [50] reported that HK2 protein content is a determinant of exercise endurance capacity in the mouse. The authors demonstrated that reducing HK content (50% HK knockout) impaired endurance and increasing HK content (HK over-expression) enhanced endurance, a finding similar to our finding with the double mutant mice *vdac1*$^{-/-}$/*vdac3*$^{-/-}$. In a previous report, the authors reported that HK2 heterozygous mice maintained on a mixed background, in which the capacity for glucose phosphorylation is diminished, have the same exercise capacity as wild type mice. However, under the stress of exercise, muscle glucose uptake was impaired in the soleus muscle [13]. Although the authors did not determine the mitochondria-bound hexokinase activity in the heterozygous muscles, their data parallel our results from the *vdac1*$^{-/-}$ mice.

In conclusion, the main finding in this report is the altered glucose clearance in VDAC1 deficient mice. While the underlying mechanism for such alteration is presently unknown and could be linked to a beta cell defect or altered glucagon secretion or altered gluconeogenesis or glycogenesis, we have demonstrated the possibility of the existence of an additional site for regulation of glucose utilization, mitochondria-bound hexokinase activity.

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